IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:
THIEL et al.

Serial No.: 09/839,796 Group: 1648

Filed: April 9, 2001 Examiner: S. Foley

For: PESTIVIRUS MUTANTS AND VACCINES CONTAINING THE SAME

DECLARATION UNDER 37 C.F.R. §1.132

Honorable Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Dr. Birgit Makoschey, declare as follows:

I am a group leader virological mammalian vaccines at Akzo Nobel N.V. (Intervet International BV) and have a Ph.D. in veterinary medicine. I am familiar with the contents of U.S. Patent Application No. 09/839,796. I have supervised a study of the safety and efficacy of 5′ deletion mutants of BVDV type I strain CP7.

This study was performed to answer two distinct questions. Firstly, does the deletion of specific sequences within the 5'untranslated region of Bovine viral diarrhea virus (BVDV) result in attenuation of the virus in the target species and secondly, do these deletion mutants protect against subsequent

challenge infection with pathogenic virus.

The deletion mutants were constructed on the basis of cp7-A5, which is an infectious clone of strain cp 7. In the first part of the study, two groups of five calves were infected both by the intranasal and the intramuscular route with deletion mutants namely with strains Δ2-31 or Δ5-57 respectively. The third group of five calves was infected with cp7-A5. The fourth group of five animals was kept as uninfected control.

Five weeks after the first infection, a challenge virus infection was performed with the BVDV type 1 strain New York. All animals of groups 1 - 3 and three animals of group 4 were challenge virus infected. The two remaining animals of group 4 were kept as uninfected challenge control.

The animals were observed for clinical signs and their body temperature was recorded after first and second infection. Samples were taken to determine white blood cell, lymphocyte and platelet counts as well as to measure viremia and neutralizing antibody titers.

RESULTS

Determination of the virulence of the 5'UTR mutants as compared to cp7-A5

Clinical reactions and body temperatures after primary infection

There were only very mild signs of respiratory disease observed after inoculation of cp7-A5 or the mutants (see table 1 for clinical scores). Similar signs were also recorded for some of the uninfected animals and the differences between groups were not significant. Therefore, no conclusions can be drawn on this parameter.

All animals infected with the cp7-A5 strain had pyrexia during one or more days, but after infection with the mutants, no elevated temperatures were recorded (individual data not shown). The average temperature in the cp7-A5 group was significantly higher than the one of the three other groups (see table1).

Leukocytes and lymphocytes counts after primary infection

The animals inoculated with cp7-A5 had a remarkable pattern in the course of white blood cell counts (see fig. 1a): the average number of leukocytes displayed a marked biphasic drop at 5 and 10 days after inoculation (dpi) , with normal or slightly increased leukocyte counts at 7 days after inoculation. From 10 days onwards till 14 days, the average leukocyte counts stayed

low. After inoculation with Δ5-57, only a slight decrease in average leukocyte counts could be observed from 7 dpi onwards and the animals inoculated with Δ2-21 had stable leukocyte counts, indicating, that the mutants were attenuated with this respect. The lymphocyte counts of all three groups remained stable after inoculation with cp7-A5 or the mutants (fig 2a).

Viremia after primary infection

After inoculation with cp7-A5, BVDV could be re-isolated from the leukocytes of all five animals and from the plasma of 3 animals during one day (see table 2). In contrast, infection with the mutants resulted only in detectable cell bound viremia in three out of five animals (Δ2-31) or one out of five animals (Δ5-57) and the duration of viremia was clearly shorter than in the cp7-A5 group. These data demonstrate, that the mutants were strongly reduced in their infectivity, thus highly attenuated.

Efficacy of the mutants to protect against secondary infection with the New York strain

Clinical reactions after secondary infection

After infection with the New York strain, only one of the three animals that were naïve at the time of infection showed clear signs of respiratory disease including an elevated body temperature (data not shown). The remaining two animals from

this group showed only very mild signs of respiratory disease, therefore, no conclusions about protection against clinical disease could be made.

White blood cell counts after infection with strain New York

The same remarkable pattern in course of the leukocyte counts as observed after infection with the cp7-A5 strain, was also detected in the three animals, that were naïve at the time of the infection with strain New York (see fig. 1b). These animals also displayed a clear drop in lymphocyte counts during the first week after infection (fig. 2b). In contrast, animals that had previously been inoculated with cp7-A5 or one of the mutants were protected against the biphasic drop in leukocyte counts and the drop in lymphocyte counts.

Viremia after infection with strain New York

Infection with the strain New York resulted in cell bound viremia during 3 to 5 days in the three animals that had not been given a primary infection, whereas all animals that had been previously infected with cp7-A5 or a mutant were completely protected against viremia (see table 2).

Testing of sera for neutralizing antibodies

All animals inoculated with cp7-A5 or the mutants developed moderate or high titers of BVDV neutralizing antibody titers.

The course of antibody titers measured against strain cp7 are depicted in figure 3. After the secondary infection, no booster effect on the neutralizing antibody titers could be detected, confirming indicating, that the animals were protected against the secondary infection. Similar results were obtained against strain New York (data not shown).

Table 1: Clinical signs and body temperatures after the first infection

Group	score		max temp	
	average	stdev	average	stdev
Δ 2-31	4.2	2.39	39.4°C	0.13
Δ 5-57	7.4	4.04	39.4°C	0.23
Cp7-A5	4.0	3.39	40.6°C	0.27
None	5.4	3.65	39.9°C	0.49

Table 2: Number of days of cell bound and cell free viremia after first and second infection

Group	Animal numbers	first infection		second infection	
		Leukocytes	Plasma	Leukocytes	Plasma
Δ 2-31	0262	1	0	0	0
	1032	0	0	0	0
	1036	1	0	0	0
	1102	2	0	0	0
	1215	0	0	0	0
Δ5-57	1031	0	0	0	0
	1211	0	0	0	0
	1273	0	0	0	0
	1642	0	0	0	0
	1650	1	0	0	0
cp7-A5	0417	4	1	0	0
	0655	3	0	0	0
	0657	5	0	0	0
	2378	5	1	0	0
	1035	2	1	0	0
challenge	0653	0	0	5	0
	1033	0	0	4	0
	1643	0	0	3	0
negative control	0653	0	0	0	0
	1028	0	0	0	0

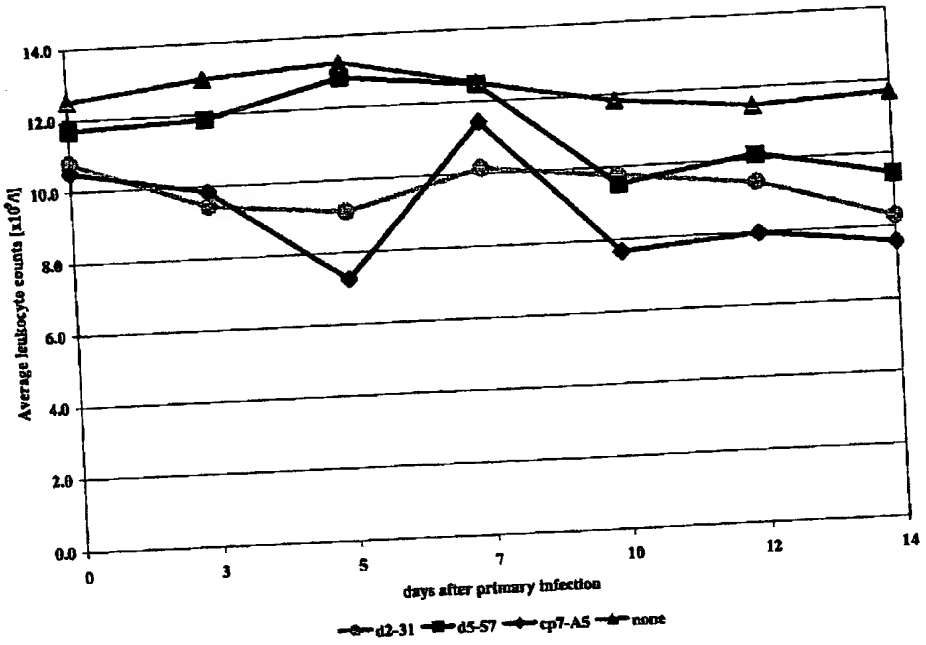

Figure 1a: Mean white blood cell counts [10^9/L] after the first infection

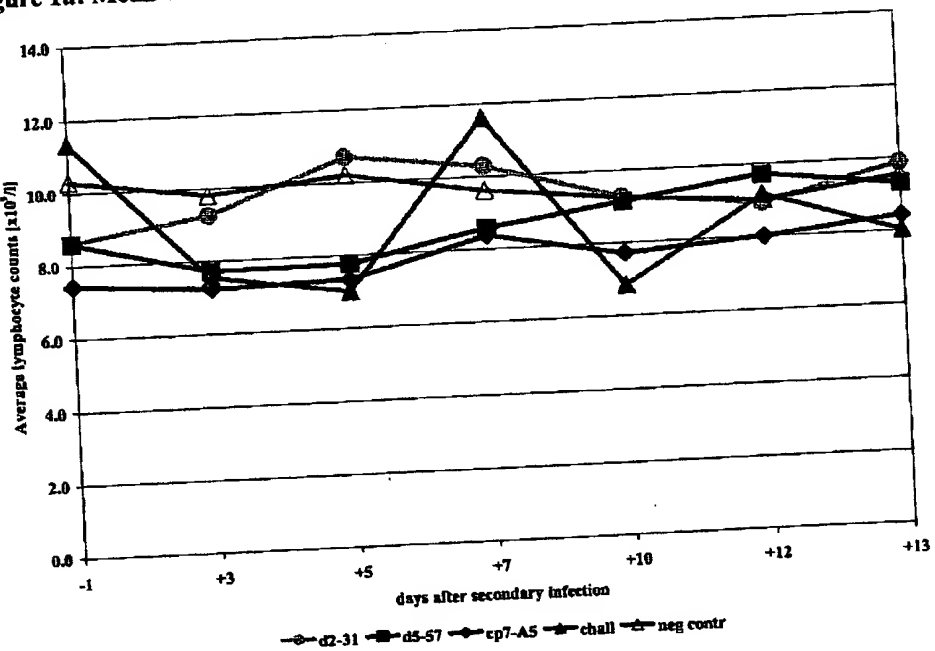

Figure 1b: Mean white blood cell counts [10^9/L] after the challenge infection

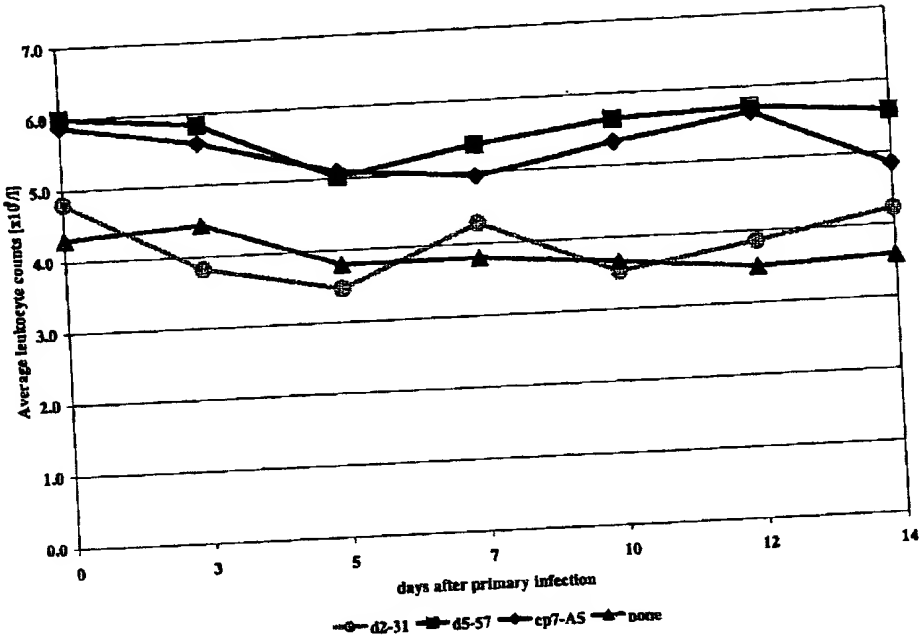

Figure 2a: Mean lymphocyte counts [10^9/L] after the first infection

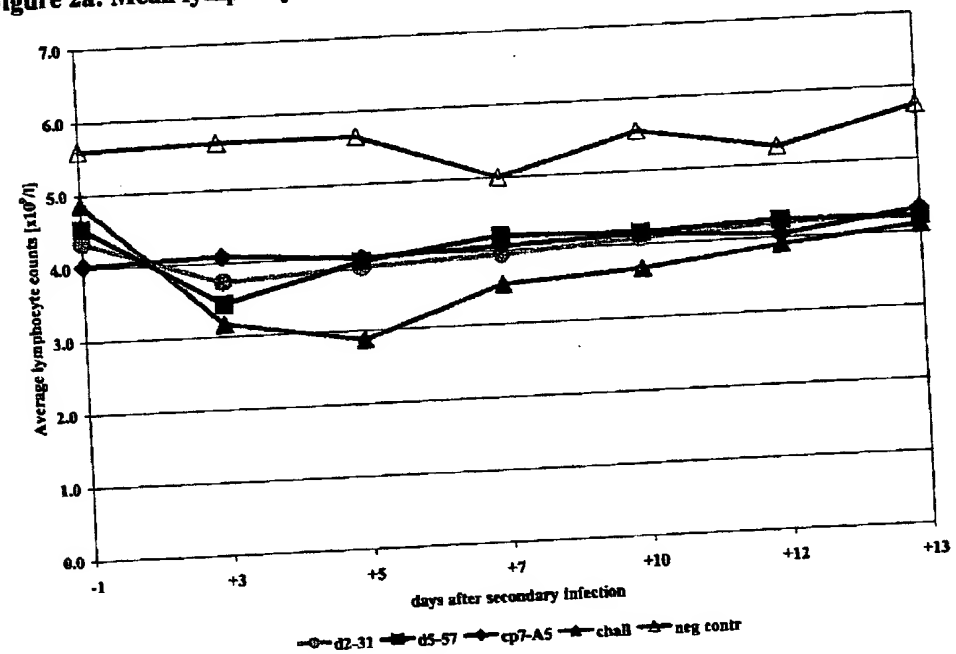

Figure 2b: Mean lymphocyte counts [10^9/L] after the challenge infection

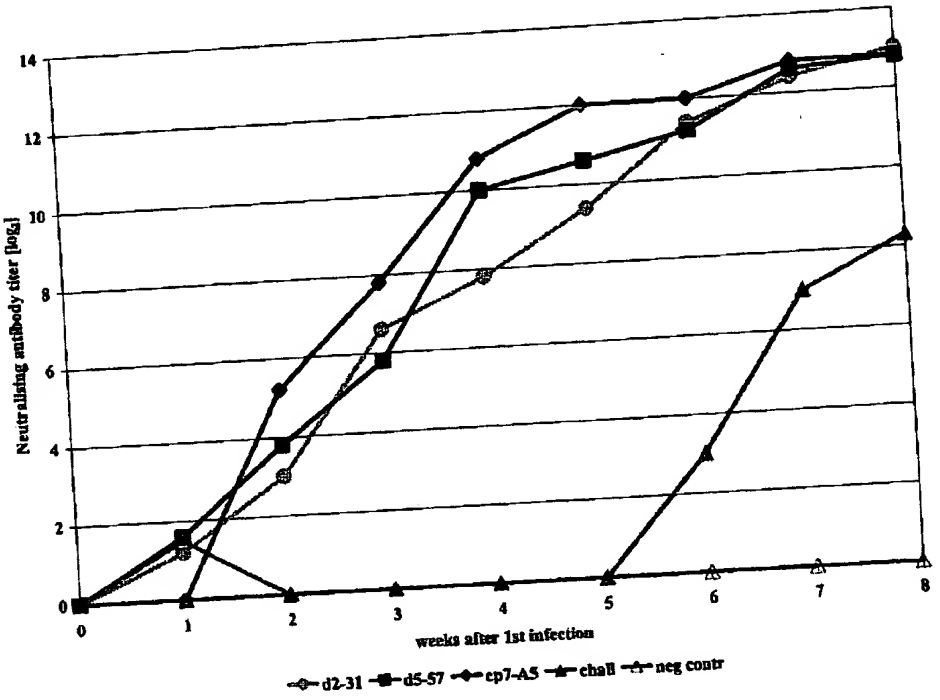

Figure 3: Neutralising antibody titers against strain cp 7

From the results obtained, a number of conclusions on the safety and the efficacy of the 5'UTR mutants can be drawn: Conclusions on the mutants were based on the comparison of the mutants with cp7-A5 which is their parent strain.

Both mutants were highly attenuated. The deletion of nucleotides 2 through 21 lead to a clear reduction of viremia and deletion of nucleotides 5 through 57 even resulted in complete lack of viremia in 4 out of 5 animals. This reduced replication of the mutants is also reflected in reduction of different clinical effects of the viruses after infection of the

animals like pyrexia or drop in leukocyte counts.

Despite the very limited replication in the host, the mutants were still able to induce sterile immunity against superinfection with the New York strain, which is the ultimate level of protection.

It can be summarized, that the 5'UTR mutants combine a good safety profile with very good efficacy and are therefore well suited as candidate live vaccines.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 17 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the captioned application or any patent issued thereon.

Makoschey

Birgit Makoschey, Ph.D.

21-Nov-2002

Date